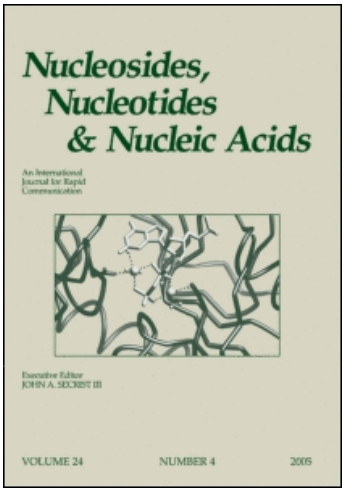

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Fluorescence Studies of Substrate Binding to Human Recombinant Deoxycytidine Kinase

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ABSTRACT

Deoxycytidine kinase (dCK), is responsible for the phosphorylation of deoxynucleosides to the corresponding monophosphates using ATP or UTP as phosphate donors. Steady-state intrinsic fluorescence measurements were used to study interaction of dCK with substrates in the absence and presence of phosphate donors. Enzyme fluorescence quenching by its substrates exhibited unimodal quenching when excited at 295 nm. Binding of substrates induced conformational changes in the protein, suggesting that dCK can assume different conformational states with different substrates and may account for the observed differences in their specificity. dCK bound the substrates more tightly in the presence of phosphate donors and UTP is the preferred phosphate donor. Among the substrates tested, the antitumour drugs gemcitabine and cladribine were bound very tightly by dCK, yielding K_d values of 0.75 and 0.8 μM, respectively, in the presence of UTP.

Key Words: Deoxycytidine kinase; Gemcitabine; Fluorescence spectroscopy.

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INTRODUCTION

Deoxycytidine kinase (dCK, EC 2:7.1.74) is important in nucleoside therapeutics since it phosphorylates many important anticancer and antiviral drugs.[1] The K_m values for nucleoside substrates are usually lower when UTP is used as phosphate donor compared to ATP.[2] dCK phosphorylates all three natural deoxyribonucleosides, dCyd, dAdo and dGuo, but with very complex kinetics.[3,4] Binding of substrates induced conformational changes in the secondary and tertiary structures of dCK[3,5] and the nature and extent of these changes varied with the substrates. In this study, we examined the effects of binding of various nucleoside substrates on the tryptophan fluorescence of dCK to determine their affinity for dCK in the absence and presence of phosphate donors. The substrates tested bound more tightly to dCK in the presence of phosphate donors and the binding affinity of dCK was greater in the presence of UTP than ATP. In the present study, for the first time we have determined the binding affinities of dCK for some of the important nucleoside analogues that are used in the treatment of cancer.

MATERIALS AND METHODS

Recombinant human dCK was prepared and purified using the pET-9d bacterial vector system (Novogen, Madison) as described previously.[5] Steady-state fluorescence measurements were made at 25°C on a Perkin-Elmer LS-55 as described.[5] Protein fluorescence was excited at 295 nm, and changes in fluorescence intensity upon ligand binding were monitored at the emission maximum (335 nm). The accessibility of the tryptophan residues (f_a) to substrates were determined from the fluorescence quenching data using the modified Stern-Volmer equation.[6]

RESULTS AND DISCUSSION

Addition of substrates to dCK resulted in quenching of protein fluorescence and, since the protein was excited at 295 nm, only tryptophan fluorescence was monitored. Fluorescence titrations with substrates were carried out as described previously.[5] Nonlinear regression analysis of the gemcitabine binding data revealed unimodal binding with a K_d value of 0.98 ± 0.04 μM. However, in an earlier study when the protein was excited at 285 nm, at which both tryptophans and tyrosines absorb, dCK exhibited two binding sites for dCyd with K_d values of 1.45 ± 0.3 and 150.8 ± 1.0 μM, for the high and low affinity sites (or states), respectively, indicating that binding to the low affinity sites perturbed certain tyrosine residues. In this work, since tryptophan residues were selectively excited, the binding of gemcitabine and other substrates to the high affinity sites was monitored in the absence and presence of phosphate donors (Table 1).

dCK bound both the pyrimidine and purine nucleoside analogues more tightly in the presence of UTP. UTP was the preferred phosphate donor, since the observed K_d values were lower in the presence of UTP as compared to the values with ATP. dCK bound the three natural deoxyribonucleosides in the following order: dCyd > dAdo > dGuo which is in agreement with earlier enzyme kinetic results.[7] Among the cytosine-containing nucleosides tested, the rank order of binding affinities for dCK with its

Table 1. Binding of ligands to the dCk in the absence and presence of phosphate donors.

	ATP		UTP	
Ligand*	K_d (μM)	K_d (μM)	K_d(μM)	f_a[†]
Gemcitabine	0.98 ± 0.05	0.85 ± 0.04	0.75 ± 0.04	0.36
dCyd	1.25 ± 0.10	0.95 ± 0.05	0.75 ± 0.05	0.34
3TC**	1.40 ± 0.15	n.d	n.d	0.33
Ara-C	1.60 ± 0.15	1.35 ± 0.10	1.20 ± 0.1	0.30
Cytidine	2.70 ± 0.20	n.d	n.d	0.26
Cladribine	0.95 ± 0.05	0.88 ± 0.04	0.80 ± 0.04	0.40
Fludarabine	1.25 ± 0.10	n.d	n.d	0.32
dAdo	1.90 ± 0.10	1.80 ± 0.10	1.40 ± 0.10	0.26
Adenosine	2.50 ± 0.20	n.d	n.d	0.24
dGuo	3.90 ± 0.20	2.05 ± 0.10	1.80 ± 0.10	0.23
dCTP	1.20 ± 0.10	n.d	n.d	0.37

*In the absence of phosphate donors. n.d. not determined.

[†]Fractional accessibility of tryptophan residues (f_a) was determined using the modified Stern-Volmer equation. (From Ref. [6].)

**3TC, L-2′3′-dideoxy-3′-thiacytidine.

substrates were; gemcitabine > dCyd > 3TC > ara-C > cytidine. For the purine nucleosides, the observed binding affinities exhibited the following order: cladribine > fludarabine > dAdo > Ado > dGuo. The feedback inhibitor dCTP also exhibited strong affinity for dCK. The accessibility of the tryptophan residues (f_a values) to the substrates as obtained from the fluorescence quenching data varied from 0.23 to 0.40 (Table 1). These results demonstrated that dCK can assume different conformational states upon binding of its various substrates, since tryptophan residues were exposed to aqueous environment to variable extents. The affinity with which dCK binds its substrates also varied. Although, dCK is known to phosphorylate these nucleoside analogues, the binding affinity (K_d) for these substrates is not known. We have demonstrated that dCK can exist in different conformational states with different affinities for its substrates; these different states may account for its broad substrate specificity.

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